

Genes Activated by *Vibrio cholerae* upon Exposure to *Caenorhabditis elegans* Reveal the Mannose-Sensitive Hemagglutinin To Be Essential for Colonization

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ABSTRACT During its life cycle, the facultative human pathogen *Vibrio cholerae*, which is the causative agent of the diarrheal disease cholera, needs to adapt to a variety of different conditions, such as the human host or the aquatic environment. Importantly, cholera infections originate from the aquatic reservoir where *V. cholerae* persists between the outbreaks. In the aquatic environment, bacteria are constantly threatened by predatory protozoa and nematodes, but our knowledge of the response pathways and adaptation strategies of *V. cholerae* to such stressors is limited. Using a temporally controlled reporter system of transcription, we identified more than 100 genes of *V. cholerae* induced upon exposure to the nematode *Caenorhabditis elegans*, which emerged recently as a valuable model for environmental predation during the aquatic lifestyle of *V. cholerae*. Besides others, we identified and validated the genes encoding the mannose-sensitive hemagglutinin (MSHA) type IV pilus to be significantly induced upon exposure to the nematode. Subsequent analyses demonstrated that the mannose-sensitive hemagglutinin is crucial for attachment of *V. cholerae* in the pharynx of the worm and initiation of colonization, which results in growth retardation and developmental delay of *C. elegans*. Thus, the surface adhesion factor MSHA could be linked to a fitness advantage of *V. cholerae* upon contact with bacterium-grazing nematodes.

IMPORTANCE The waterborne diarrheal disease cholera is caused by the bacterium *Vibrio cholerae*. The facultative human pathogen persists as a natural inhabitant in the aquatic ecosystem between outbreaks. In contrast to the human host, *V. cholerae* requires a different set of genes to survive in this hostile environment. For example, predatory micrograzers are commonly found in the aquatic environment and use bacteria as a nutrient source, but knowledge of the interaction between bacterivorous grazers and *V. cholerae* is limited. In this study, we successfully adapted a genetic reporter technology and identified more than 100 genes activated by *V. cholerae* upon exposure to the bacterium-grazing nematode *Caenorhabditis elegans*. This screen provides a first glimpse into responses and adaptational strategies of the bacterial pathogen against such natural predators. Subsequent phenotypic characterization revealed the mannose-sensitive hemagglutinin to be crucial for colonization of the worm, which causes developmental delay and growth retardation.

KEYWORDS MSHA, RIVET, bacterial grazing, bacterivorous, chitin, development, environment, growth retardation, nematodes, nutrition, predator model, survival

The Gram-negative bacterium *Vibrio cholerae* is the causative agent of the severe human diarrheal disease cholera, which is now endemic in many areas of the African and Asian continents (1). Lately, an increasing frequency of major epidemics,

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 This study provides a first glimpse into responses & adaptational strategies of *Vibrio cholerae*, causative agent of cholera, against bacterium-grazing nematode *C. elegans*.

including, e.g., Zimbabwe (2009), Haiti (2010), and Yemen (ongoing), has drawn attention from the public. However, these local outbreaks represent just the tip of the iceberg of this devastating disease with an overall global burden estimated to be 3 to 5 million cases and up to 130,000 deaths per year (2). Particularly children show a high mortality rate in developing countries, where diarrheal diseases remain the second most common cause of death.

Hallmarks of the life cycle of the clinically relevant *V. cholerae* isolates are the transitions between two dissimilar habitats, i.e., as a natural inhabitant of aquatic ecosystems and as a pathogen in the human gastrointestinal tract (3, 4). The fast adaptation of *V. cholerae* between the alternative lifestyles is remarkable and represents a key feature for the transition fitness between the different stages of the *V. cholerae* life cycle, allowing rapid spread of cholera. At the beginning of an outbreak, a relatively high infection dose of 10^6 to 10^9 CFU is required to cause disease (5). However, after passage through the host *V. cholerae* exits in a transient hyperinfectious state. For reinfections of humans within this period, the infectious dose can drop down to 10^2 to 10^3 CFU (6, 7). As outbreaks generally originate from the aquatic reservoir, a better understanding of the whole life cycle of *V. cholerae* with an emphasis on the persistence and survival of *V. cholerae* in the aquatic ecosystems has become a major target for the development of new therapeutic strategies and the containment of the disease (1, 2, 5, 8, 9).

V. cholerae spends much of its life cycle outside the host in estuarine and coastal aquatic reservoirs with a geographical range from tropics to temperate water worldwide. The capability of *V. cholerae* to survive in many different environmental niches requires adaptation to a number of fluctuating conditions, including temperature shifts, osmotic stress, and nutrient limitation (10). To survive these challenges, *V. cholerae* employs a number of strategies, including formation of biofilms on abiotic and biotic surfaces, the conversion into a viable but nonculturable (VBNC) state under unfavorable conditions, and the acquisition and storage of nutrients.

In addition, bacterivorous predators like free-living protozoa and nematodes are a constant threat for environmental bacteria (11). Vice versa, bacteria have evolved protective responses to such biological stressors. In order to study the interactions between *V. cholerae* and bacterium-grazing invertebrates, the nematode *Caenorhabditis elegans* has been successfully established as a natural predator model. So far, two secreted effectors of *V. cholerae*, the protease PrtV and the hemolysin HlyA, have been identified to cause lethality or developmental delay in the nematode *C. elegans* and thereby contribute to protection from natural predator grazing (12, 13). A microarray study compared the host gene expression response of *C. elegans* exposed to *V. cholerae* wild type (WT) and an *hlyA* mutant (14). Several genes specifically induced by the worm in response to HlyA have been identified, including pathways previously implicated in innate immune response pathways (14). Furthermore, a recent study demonstrated that the quorum-sensing molecule CAI-1 of *V. cholerae* is recognized by *C. elegans* as an attractant via the AWC^{ON} neuron (15). These studies are the first indications of defined responses by *C. elegans* upon contact with *V. cholerae* and provide a first glimpse into cross-kingdom interactions.

In contrast, transcriptional changes taking place in *V. cholerae* upon contact with such bacterium-grazing nematodes have not been investigated. In this study, the recombination-based *in vivo* expression technology (RIVET) was adapted to identify more than 100 *V. cholerae* genes activated upon exposure to the nematode, including those for the biogenesis of the mannose-sensitive hemagglutinin (MSHA). Subsequent mutagenesis and phenotypic analyses revealed MSHA to be an important factor enabling *V. cholerae* to colonize the pharynx of *C. elegans*.

RESULTS

Identification of genes activated upon exposure to *C. elegans* (*aec*). To identify genes of *V. cholerae* activated upon exposure to the bacterium-grazing nematode *C. elegans* (*aec*), we screened a library with approximately 12,000 random transcrip-

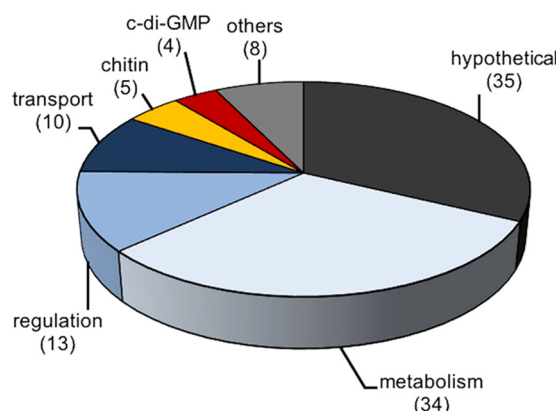


FIG 1 Functional distribution of genes activated upon exposure to *C. elegans* (*aec* genes). Shown are *aec* genes identified with recombinase-based screening technology, allocated in functional groups by their proposed function according to KEGG (<http://www.genome.jp/kegg/>) (43–45). The number of *aec* genes in the respective group is indicated in parentheses.

tional gene-*tnpR* fusions, previously used to identify genes induced *in vivo* or in biofilm (16, 17). We recovered and sequenced gene-*tnpR* fusions of ~200 resolved strains, which resulted in the identification of 109 plus-stranded fusions to different genes (see Table S1 in the supplemental material). Figure 1 provides an overview of the *aec* genes divided into functional groups: 35 of the *aec* genes are hypothetical, 34 are implicated in metabolism, 13 are predicted to be involved in signaling and regulatory pathways, 10 are involved in transport, 5 have a role in chitin utilization or attachment, 4 are involved in c-di-GMP metabolism, and 8 do not fit into one of these groups and are therefore named “others.” In addition, a gene ontology analysis was performed using PANTHER (<http://pantherdb.org>), which supports enrichment analysis using pathway classifications from the reactome pathway database (18). Using the “biological process” classification, which groups the genes into nine subgroups (biological regulation, cellular component organization or biogenesis, cellular processes, localization, locomotion, metabolic process, multicellular organismal processes, reproduction, and response to stimulus), the categories “metabolic processes” ($P = 0.03$) and “biological regulation” ($P = 0.01$) were found to be significantly overrepresented in the identified *aec* genes with regard to their abundance in the *V. cholerae* genome.

Ten representative gene-*tnpR* fusions were selected for a validation process and used to reconstruct unresolved strains. Resolution frequencies of the reconstructed strains were determined under *in vitro* control conditions (growth on nematode growth medium [NGM] agar plates without *C. elegans*) as well as after exposure to *C. elegans* to determine the accuracy of the screen (see Materials and Methods for details). A significantly higher resolution frequency upon exposure to *C. elegans* than that for the control condition was observed for 7 out of 10 reconstructed gene-*tnpR* fusion strains (Fig. 2), which corresponds to an accuracy rate of approximately 70%. Notably, several of the identified *aec* genes are important for attachment to chitin or its utilization as a nutrient source (19). Two (VC0412 and VCA0027) could be verified to be bona fide *aec* genes in the validation process. Especially the VC0412-*tnpR* fusions demonstrated a robust increase in resolution frequency upon exposure to *C. elegans* over that for the control conditions. VC0412 belongs to the *msh* operon essential for synthesis of the mannose-sensitive hemagglutinin (MSHA) type IV pilus (20). To investigate the role of the MSHA pilus upon contact with *C. elegans* in more detail, we constructed the MSHA-negative $\Delta mshA$ mutant with an in-frame deletion of VC0409, encoding the major subunit of the pilus (21, 22).

MSHA promotes attachment of *V. cholerae* in the pharynx of *C. elegans*. The MSHA pilus facilitates surface adhesion of *V. cholerae*, which is a prerequisite for successful biofilm formation on chitinous surfaces provided by zooplankton in the

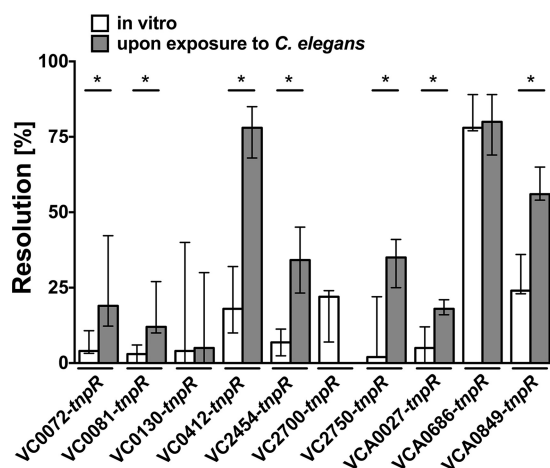


FIG 2 Validation of *aec* genes. Shown are the median resolution frequencies of reconstructed *tnpR*-fusion strains (indicated on the x axis) under *in vitro* conditions (open bars) and upon exposure to *C. elegans* (gray bars). The error bars indicate the interquartile ranges. Each data set consists of at least 6 independent experiments. A significant increase in the resolution frequency between *in vitro* conditions and upon exposure to *C. elegans* is indicated by an asterisk (*, $P < 0.05$ by Mann-Whitney U test).

aquatic environment (19). Chitin is present on nematode surfaces, and a recent study demonstrated that the polymer is an indispensable component of the chitin decoration of the *C. elegans* pharynx localized to specific regions of the lumen wall (23). Taking into account the impact of MSHA on development and the identification of several genes of the chitin utilization program, it could be hypothesized that *V. cholerae* can colonize the pharynx, with MSHA being a relevant factor for attachment. The impact of MSHA on a potential colonization of *V. cholerae* in the pharynx of *C. elegans* was investigated by fluorescence microscopy. *C. elegans* was cultivated on either the green fluorescent protein (GFP)-labeled *V. cholerae* wild type (WT) or the $\Delta mshA$ mutant before the nematodes were analyzed by microscopy. A comprehensive evaluation of microscopic images with more than 100 worms revealed that almost all worms grazing on *V. cholerae* WT pgfp (constitutive expression of GFP from a plasmid) exhibited a fluorescent signal in the pharynx, while only approximately 50% of worms grazing on $\Delta mshA$ pgfp showed a signal above the limit of detection (Fig. 3A). To prove that the visualized bacteria are indeed attached to the pharyngeal luminal surface, worms were passaged for another 2 h via NGM agar plates seeded with *Escherichia coli* OP50 without GFP expression to remove luminal or loosely attached bacteria inside the worms. After this passage, approximately 75% of the worms grazing on *V. cholerae* WT pgfp still showed a detectable fluorescent signal, while only in 25% of worms grazing on $\Delta mshA$ pgfp was a signal detected (Fig. 3B). Representative images showing the fluorescent intensity and signal localization in worms grazing on *V. cholerae* WT pgfp or $\Delta mshA$ pgfp after a 2-h passage on OP50 are provided in Fig. 4. While in worms fed on *mshA* mutants no or only low fluorescence was observed, nematodes grazing on GFP-labeled *V. cholerae* WT showed a reproducible signal in the pharynx. To obtain quantitative data, colonization levels were determined for worms grazing on *V. cholerae* WT or $\Delta mshA$ mutant after the 2-h passage on *E. coli* OP50 (Fig. 3C). Despite a relatively high variation in colonization levels of WT and $\Delta mshA$ strains, worms grazing on *V. cholerae* WT showed significantly higher colonization than worms fed with the $\Delta mshA$ mutant. *C. elegans* grazing on *V. cholerae* WT showed a median colonization level of $\sim 2 \times 10^3$ CFU per worm, while nematodes grazing on the $\Delta mshA$ mutant exhibited a median colonization level of $\sim 2 \times 10^1$ CFU per worm. Taken together, the results indicate that MSHA is important for adherence of *V. cholerae* in the pharynx of *C. elegans*, which is an important step for successful colonization.

MSHA does not contribute to the lethality of *C. elegans*. As *V. cholerae* has been shown to cause lethality in *C. elegans* via secreted effectors, i.e., the hemolysin HlyA or

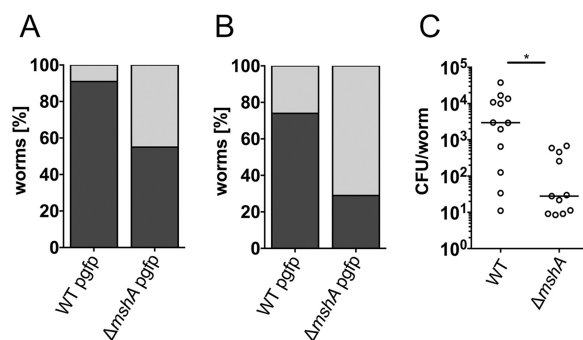


FIG 3 MSHA is crucial for colonization of *C. elegans* by *V. cholerae*. (A and B) Shown is the percentage of worms with detectable signal (dark gray) or no detectable signal (light gray) in the pharynx after grazing on *V. cholerae* WT pgfp or $\Delta mshA$ mutant pgfp before (A) or after (B) passage via *E. coli* OP50 for 2 h. At least 100 worms of each data set were analyzed. In both cases (A and B), the distributions of worms with and without signal are significantly different between nematodes grazing on *V. cholerae* WT pgfp and those grazing on $\Delta mshA$ mutant pgfp (*, $P < 0.05$ by Fisher exact test). (C) Shown are the colonization levels of worms grazing on *V. cholerae* WT or $\Delta mshA$ mutant after passage via *E. coli* OP50 for 2 h. Each circle represents the colonization level determined from 7 to 12 worms pooled for homogenization. The CFU were divided by the number of worms present in the respective homogenized pool to retrieve the CFU per worm (for details, see “Quantification of bacterial colonization” in Materials and Methods). The horizontal bar indicates the median of each data set. A significant difference is indicated by an asterisk (*, $P < 0.05$ by Mann-Whitney U test).

the protease PrtV (12, 13), a potential contribution of MSHA to cause reduced longevity of *C. elegans* was investigated. Worms were raised on *E. coli* OP50 to reach the L4 stage before they were transferred onto plates harboring lawns of either *E. coli* OP50 or *V. cholerae* WT or $\Delta mshA$ mutant. In accordance with previous results (12, 13), we observed a marked decrease in the survival rate for the nematodes grazing on *V. cholerae* WT, with a median survival of approximately 5 days, compared to growth on *E. coli* OP50, with a median survival of approximately 14 days. As the worms exhibited similar survival dynamics while grazing on *V. cholerae* WT and on the $\Delta mshA$ mutant (Fig. S1), MSHA does not seem to be a dominant factor causing lethality in *C. elegans*.

MSHA contributes to developmental delay and growth retardation of *C. elegans*. Although survival was not altered between the *V. cholerae* strains, a disparity in

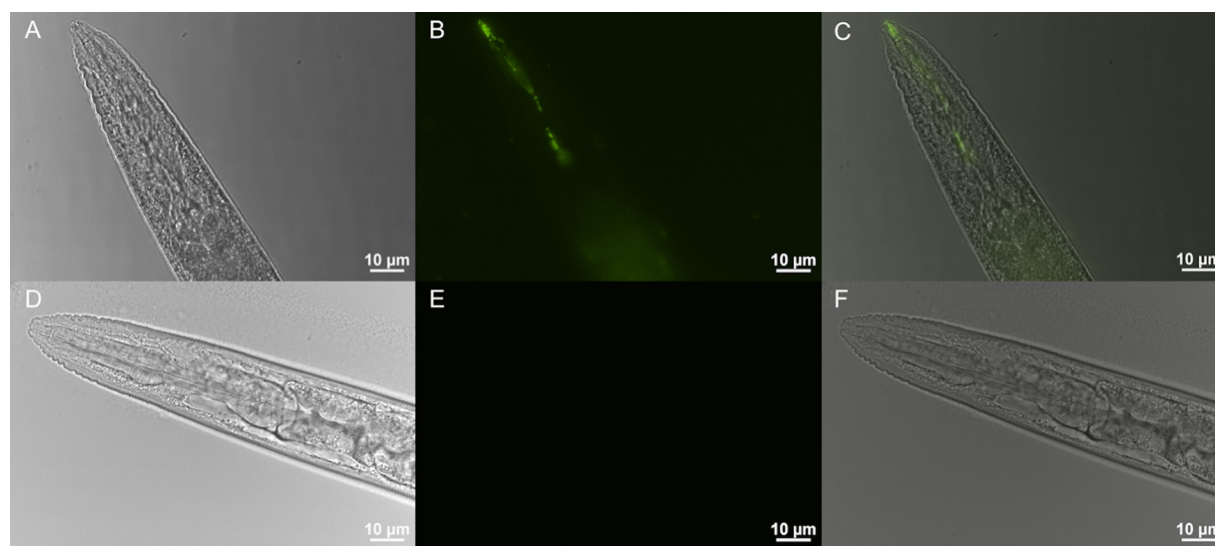


FIG 4 Localization of *V. cholerae* in the pharynx of *C. elegans* depends on MSHA. Representative epifluorescence microscopy images of the anterior parts, including the pharynx of worms cultivated on plates with *V. cholerae* WT pgfp (A to C) or *V. cholerae* $\Delta mshA$ pgfp (D to F) after a passage via *E. coli* OP50 for 2 h. GFP signal was detected in the green channel (excitation of 400 nm, emission of 500 to 550 nm). Shown are bright-field images (A and D), fluorescent images (B and E), and merged images (C and F).

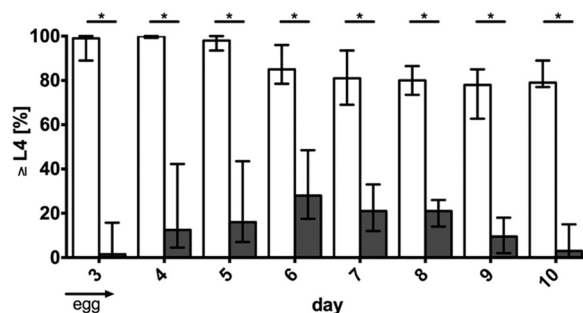


FIG 5 *V. cholerae* causes developmental delay in *C. elegans*. Development of *C. elegans* cultured on NGM agar plates containing *E. coli* OP50 (open bars) or *V. cholerae* WT (gray bars) was monitored for 10 days starting from egg stage on day 0. Shown are the median percentages of nematodes in L4 or later stages for day 3 to 10. The error bars indicate the interquartile ranges. Each data set consists of at least 10 independent experiments with an average of 30 worms per experiment. An asterisk above the respective data sets indicates a significant difference between the development of worms grazing on *E. coli* OP50 and those grazing on *V. cholerae* WT (*, $P < 0.05$ by Mann-Whitney U test).

the size of worms grazing on different bacterial strains throughout the routine microscopic evaluation of the survival assays was observed. This prompted us to analyze if MSHA of *V. cholerae* might be a crucial factor for developmental delay and reduced growth of *C. elegans*. Adult worms were allowed to lay eggs for a defined amount of time on plates harboring lawns of either *E. coli* OP50 or *V. cholerae* strains (see Materials and Methods for details). Subsequently, adult worms were removed and the development of *C. elegans* eggs to the L4 stage or beyond was monitored. In order to reveal the dynamics, development of *C. elegans* grazing on *E. coli* OP50 and *V. cholerae* WT was monitored for 10 days (Fig. 5). Almost all nematodes grazing on *E. coli* OP50 reached the L4 stage within 3 to 4 days. Within the following days, the percentage of worms in L4 or later stages declined modestly, which can be explained by the increasing death rate. In contrast to *E. coli*, the percentage of *C. elegans* in L4 or later stages was significantly lower for worms grazing on *V. cholerae* WT throughout the entire assay. In the case of *V. cholerae*, the number of worms in L4 or later stages peaked on day 6, followed by a steady decline with only a few worms surviving at day 10.

To exclude the possibility that *E. coli* OP50 provides better nutrition than *V. cholerae*, which might result in the observed developmental delay, the assay was repeated with heat-killed bacteria. Worms grazing on heat-killed *V. cholerae* WT or *E. coli* OP50 showed similar development, which was comparable to that of nematodes grazing on living *E. coli* OP50 (data not shown). Thus, *V. cholerae* seems to be an adequate nutrition source for *C. elegans*. In summary, development of nematodes cultured on living *V. cholerae* WT compared to those on *E. coli* OP50 was delayed and significantly reduced.

Based on the developmental dynamics of nematodes grazing on *V. cholerae* WT, days 4, 5, and 6 were chosen for further comparative analyses of the WT and $\Delta mshA$ strains (Fig. 6A). For all time points, a significantly higher number of nematodes grazing on the $\Delta mshA$ strain reached the L4 stage compared to worms cultured on *V. cholerae* WT. While the presence of an empty vector in *V. cholerae* WT or the $\Delta mshA$ mutant had no effect on the observed differences in development of *C. elegans*, the enhanced development of worms grazing on the $\Delta mshA$ mutant was significantly decreased again upon worm cultivation on the $\Delta mshA$ mutant expressing *mshA* in *trans* on a complementation plasmid (Fig. 6B). Any effects on development by isopropyl- β -thiogalactopyranoside (IPTG) and ampicillin (Ap) can be excluded, as worms grazing on either regular NGM plates seeded with *E. coli* OP50 or NGM plates supplemented with IPTG/Ap and seeded with *E. coli* OP50 harboring an empty vector showed comparable developmental dynamics to the L4 stage (Fig. S2). Thus, the observed differences in *C. elegans* development can be attributed to the respective *V. cholerae* strain used.

Furthermore, the length of the worms and their colonization with *V. cholerae* were comprehensively analyzed on day 4. Representative microscopic images of worms

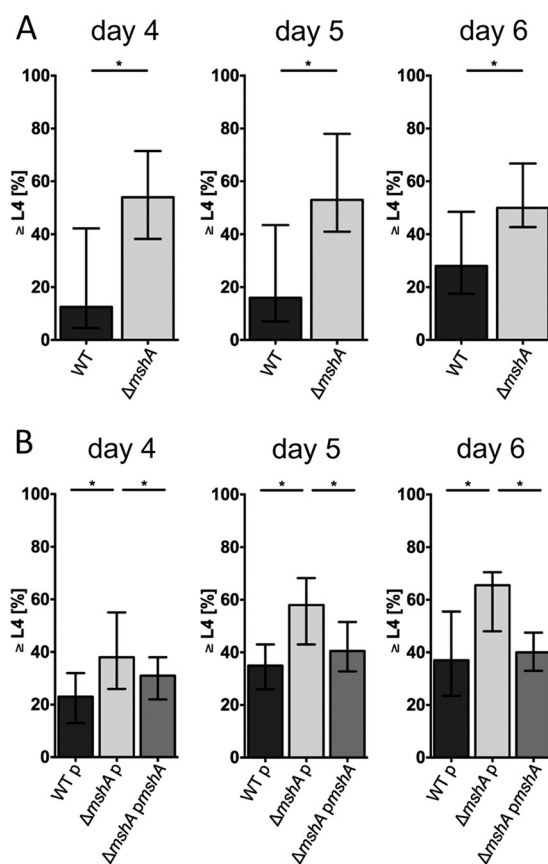


FIG 6 MSHA contributes to developmental delay of *C. elegans* grazing on *V. cholerae*. Development of *C. elegans* grazing on different *V. cholerae* strains (as indicated on the x axis) was monitored for 10 days starting from egg stage on day 0. Shown are the median percentages of nematodes in L4 or later stages for days 4, 5, and 6. The error bars indicate the interquartile ranges. Each data set consists of at least 8 independent experiments with an average of 30 worms per experiment. (A) Significant differences in the development of worms grazing on *V. cholerae* WT compared to those grazing on the $\Delta mshA$ mutant are indicated with an asterisk (*, $P < 0.05$ by Mann-Whitney U test). (B) Significant differences in the development of worms grazing on the $\Delta mshA$ mutant with empty vector compared to those grazing on *V. cholerae* WT with empty vector or complementation strain are indicated with an asterisk (*, $P < 0.05$ by Kruskal-Wallis test followed by *post hoc* Dunn's multiple-comparison test).

grazing on *E. coli* OP50 or the *V. cholerae* WT or $\Delta mshA$ strain are presented in Fig. 7A to C. *C. elegans* worms grazing on *V. cholerae* WT were significantly smaller than *E. coli* OP50-cultured worms (Fig. 7D). Consistent with the developmental assay, nematodes cultivated on the $\Delta mshA$ strain were significantly larger than those cultivated on *V. cholerae* WT, demonstrating that the absence of MSHA partially negates the developmental delay and growth retardation of *C. elegans* grazing on *V. cholerae*. In addition, a GFP-expressing variant of *V. cholerae* WT was used to analyze the spatial distribution in the worm at day 4 of the developmental assay (Fig. S3). A marked heterogeneity in the localization could be observed. The majority of worms showed detectable levels in the pharynx (Fig. S3), which is consistent with the localization in L4-synchronized worms cultivated for 1 day on *V. cholerae* WT pgfp (Fig. 4). However, several worms also exhibited fluorescence in the lower gut (Fig. S3F and I), suggesting that *V. cholerae* can spread to other parts of the gut at later time points. In some cases, the fluorescent signal seemed to be blurred. Thus, we cannot exclude the possibility that some fluorescence originates from GFP released from lysed *V. cholerae*. It should be noted that the analysis was hampered by the fragility of worms, which resulted in lysis and disintegration of the worms. An example of a damaged worm is given in Fig. S3J, K, and L. The prolonged cultivation on *V. cholerae* obviously causes the worms to become highly fragile for preparation for microscopy. Finally, bacterial colonization of *C. elegans*

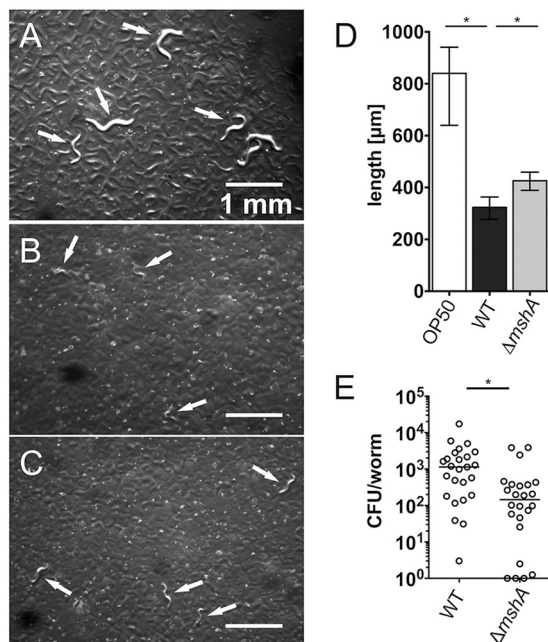


FIG 7 MSHA contributes to growth defect of *C. elegans* grazing on *V. cholerae*. (A to C) Images of plates with *C. elegans* grazing on *E. coli* OP50 (A) and *V. cholerae* WT (B) or $\Delta mshA$ (C) strain taken on day 4 of the developmental assay. Arrows mark representative worms on the plate. (D) Median length of *C. elegans* grazing on *E. coli* OP50 (open bar) or *V. cholerae* WT (dark gray bar) or $\Delta mshA$ mutant (light gray bar). The error bars indicate the interquartile ranges. Significant differences between the data sets are indicated with an asterisk (*, $P < 0.05$ by Kruskal-Wallis test followed by *post hoc* Dunn's multiple-comparison test). (E) Colonization levels on day 4 of the developmental assay of worms which have been grazing on either *V. cholerae* WT or the $\Delta mshA$ mutant. Each circle represents the colonization level determined from 7 to 12 worms pooled for homogenization. The CFU were divided by the number of worms present in the respective homogenized pool to retrieve the CFU per worm (for details, see "Quantification of bacterial colonization" in Materials and Methods). The horizontal bar indicates the median of each data set. A significant difference is indicated by an asterisk (*, $P < 0.05$ by Mann-Whitney U test).

grazing for 4 days on the *V. cholerae* WT or $\Delta mshA$ strain was quantified by CFU plating. Worms grazing on *V. cholerae* WT also showed significantly higher colonization than worms fed with the $\Delta mshA$ mutant (Fig. 7E), which is consistent with the colonization data for L4-synchronized worms (Fig. 3C). The colonization level observed on day 4 of the developmental assays was slightly lower than that of L4-stage worms, which might be due to the delayed development and growth retardation of worms fed on *V. cholerae*.

DISCUSSION

It has become evident that clinically relevant *V. cholerae* persists in the aquatic reservoir between devastating outbreaks (9, 10). It is essential that bacterial load is maintained at a certain level sufficient to cause a new outbreak. This requires several survival strategies of *V. cholerae* to cope with the harsh conditions of an aquatic lifestyle, facing, for example, temperature shifts, changes in osmolarity, and nutrient limitation. Biofilm formation, chitin utilization, and the entrance into a VBNC status are known mechanisms of *V. cholerae* to enhance persistence in the aquatic reservoir. Especially during colder periods, the sediments of the aquatic ecosystem act as a reservoir for *V. cholerae* (24, 25). Interestingly, bacterivorous nematodes are highly abundant in the sediment, which emphasizes that *V. cholerae* is under high grazing pressure and top-down control by these predators (10, 24, 26–28). Recent studies introduced the nematode *C. elegans*, which has become an important model organism for genetic and developmental studies, as a natural predator model for *V. cholerae* (12, 13). This nematode is an ideal choice as it can be easily maintained under laboratory

conditions with a variety of mutants and assays available. Both abovementioned studies demonstrated that *V. cholerae* can reduce the life span of *C. elegans* and identified two secreted effectors, the protease PrtV and the hemolysin HlyA, which contribute to the observed lethality (12, 13). These reports imply that the facultative human pathogen has evolved responses and defense mechanisms to counteract bacterivorous nematodes.

In order to gain insights into these bacterial strategies, we focused on the identification of *V. cholerae* genes upregulated upon exposure to *C. elegans* in the present study. Therefore, we used the well-established resolvase-based reporter gene system RIVET, which allows detection of spatiotemporal gene expression even in diverse populations (16, 29–31). In contrast to alternative techniques like microarrays, transcriptome sequencing (RNA-seq), or reverse transcription-quantitative PCR (qRT-PCR), this single-cell-based expression technology avoids purification of bacterial RNA in decent quality and quantity, requires relatively small amounts of bacteria, and even allows the identification of genes that are only transiently induced, as the excision of the reporter gene cassette is irreversible. Thus, the resolvase-based reporter gene system is ideally suited for a first study identifying *V. cholerae* genes in *C. elegans*. It should be noted that the presented screen does not claim to be saturated. Consequently, several other upregulated genes might have been missed and still remain to be identified.

In total, we were able to identify 109 *aec* genes. An advantage of the resolvase-based reporter gene system is the ability to recover the integrated suicide plasmids harboring the induced *tnpR* fusion and to subsequently reconstruct the original unresolved strain. These reconstructed strains can be tested for their resolution frequency under control conditions as well as during colonization of *C. elegans*. A validation revealed that 7 out of 10 reconstructed *tnpR* fusion strains showed a significantly higher resolution frequency during colonization of *C. elegans* than under the control condition. This corresponds to an acceptable accuracy rate of 70%.

Gene ontology analysis revealed that the genes involved in metabolic processes and biological regulation are enriched within the *aec* genes, indicating that *V. cholerae* alters its metabolism and regulatory pathways upon exposure to the worm. Among the identified *aec* genes were 5 relevant for adhesion to and degradation of chitin. Two of these, VCA0027, encoding a putative chitinase, and VC0414, encoding a biogenesis protein of the MSHA type IV pilus, were included in the validation and demonstrated a significant induction upon exposure to *C. elegans*. Within this study, we selected the MSHA type IV pilus for further characterization and analyzed its impact on longevity, growth, and development of *C. elegans*, which are affected by *V. cholerae*. Based on the data presented here, MSHA has no effect on physiology of the worm. Instead, MSHA has previously been shown to be important for the first steps in biofilm formation by facilitating adherence to abiotic surfaces, like borosilicate, as well as biotic surfaces, like zooplankton and crab shells (19, 32, 33). In concordance with these reports, we can demonstrate that MSHA promotes attachment and colonization of the *C. elegans* pharynx, which contains chitin on interior regions (34). Based on the current data, it is unclear whether MSHA specifically interacts with chitin or enhances chitin adherence in a surface chemistry-independent manner. Meibom et al. suggested that MSHA could also facilitate adherence to the epicuticle, which naturally covers biotic chitinous surfaces and therefore represents the outermost layer (35). Such an epicuticle would also be present in the pharynx of *C. elegans*, thought to be secreted by pharyngeal gland cells (http://www.wormbook.org/chapters/www_cuticle/cuticle.html). In fact, a deletion mutant of *gbpA*, encoding an alternative chitin-binding protein with specificity for *N*-acetylglucosamine oligosaccharides (36), was also analyzed in this study but showed no difference for *C. elegans* growth or development compared to the *V. cholerae* WT (see Fig. S4 in the supplemental material). Together, these results could indicate that initial attachment of *V. cholerae* in the pharynx is chitin independent but that the interaction could be reinforced subsequently in a chitin-dependent manner.

How can MSHA impact growth and development of *C. elegans* without affecting

longevity? Lethality for *C. elegans* by *V. cholerae* can be attributed to at least two secreted effectors, PrtV and HlyA. One could speculate that they act very potently and/or fast on *C. elegans*. As both effectors are secreted, they could also be constantly ingested by *C. elegans* from the medium in addition to the amounts secreted by *V. cholerae* colonizing the worm. Thus, a stable colonization by *V. cholerae* resulting in a long exposure to the effectors is not required to reduce the longevity of *C. elegans*. Alternatively, *V. cholerae* expresses PrtV and HlyA independently of *C. elegans* and can affect the worm directly upon first contact. Notably, neither *prtV* nor *hlyA* was identified as an *aec* gene. While lethality is a toxigenic effect, delays in development might be due to poor nutrition. Bacterial colonization in the pharynx might block ingestion of food. Alternatively, colonizing *V. cholerae* could deplete specific nutrients which are essential for *C. elegans* development. Furthermore, at least one annotated chitinase (VCA0027) has been identified and validated as a bona fide *aec* gene. Thus, it can be hypothesized that the chitin utilization program of *V. cholerae* is induced upon exposure to *C. elegans*. This will slowly but steadily result in chitin degradation on the pharyngeal luminal surfaces and thereby affect the growth and development of the worm. Future studies are currently directed to dissecting the role of the five predicted chitinases encoded by *V. cholerae*.

Altogether, this study provides a first glimpse into the response of *V. cholerae* upon exposure to *C. elegans*. In addition, MSHA was characterized as a crucial factor for attachment and colonization of the *C. elegans* pharynx, which results in growth retardation and developmental delay of *C. elegans*.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Bacterial strains and plasmids used in this study are listed in Table S2 in the supplemental material; oligonucleotides are listed in Table S3. *V. cholerae* AC53, a spontaneous streptomycin (Sm)-resistant mutant of the clinical isolate E7946 (O1 El Tor Ogawa), was used as the wild-type (WT) strain. *E. coli* strain OP50 served as a general food source for *C. elegans*, while *E. coli* strains DH5 α *pir* and SM10 λ *pir* were used for genetic manipulations. If not noted otherwise, bacterial strains were cultured in Luria-Bertani (LB) broth or on LB broth agar plates with aeration at 37°C. Antibiotics and other supplements were used in the final concentrations indicated: streptomycin (Sm), 100 μ g/ml; kanamycin (Kn), 50 μ g/ml; Ap, 100 μ g/ml or 50 μ g/ml in combination with a second antibiotic and in all assays using NGM agar plates; chloramphenicol (Cm), 30 μ g/ml (*E. coli*) or 2 μ g/ml (*V. cholerae*); IPTG, 0.5 mM; glucose (Glc), 0.2%; sucrose (Suc), 10%; 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-Gal), 30 μ g/ml.

***Caenorhabditis elegans* maintenance.** *C. elegans* strains Bristol N2 and SS104 (see Table S2) were obtained from the *Caenorhabditis* Genetics Center and routinely maintained at 24°C (Bristol N2) or 16°C (SS104) on nematode growth medium (NGM) agar plates with *E. coli* strain OP50 by standard methods (37, 38). *C. elegans* strain Bristol N2 was used for the RICET screen, while *C. elegans* strain SS104, a temperature-sensitive mutant that produces progeny at temperatures of $\leq 16^\circ\text{C}$ but not at temperatures of $\geq 24^\circ\text{C}$, was used for all other experiments to allow synchronization of the worms. Synchronization was achieved by transferring *C. elegans* SS104 cultivated at 16°C onto NGM agar plates for 2 h. After 2 h of egg laying, adult worms were removed, and incubation of the plates at 24°C allowed the hatched progeny to reach the L4 stage within 3 days.

Genetic manipulations and construction of plasmids. The isolation of chromosomal DNA, PCRs, the purification of plasmids or PCR products, the construction of suicide and expression plasmids, and the subsequent generation of deletion mutants were carried out as described previously (39). QIAquick gel extraction and QIAquick PCR purification kits (Qiagen) were used for purifying PCR products and digesting plasmid DNA. PCRs for subcloning were carried out using the Q5 high-fidelity DNA polymerase (New England BioLabs [NEB]), while *Taq* DNA polymerase (NEB) was used for all other PCRs. The Δ *mshA* and Δ *gbbA* in-frame deletion mutants were constructed according to the method of Donnenberg and Kaper (40) using the suicide vector pCVD442. The respective suicide vectors pCVD442 Δ *mshA* and pCVD442 Δ *gbbA* were constructed by PCR amplification of approximately 800-bp fragments, representing upstream and downstream regions of the gene of interest, using the oligonucleotide pairs y-x₁ and y-x₂ or y-x₃ and y-x₄, where y represents the gene (*mshA* or *gbbA*) and x represents the restriction site/enzyme used (Table S3). These upstream/downstream fragments were digested with the appropriate restriction enzyme indicated by the name of the oligonucleotide and ligated into an identically digested suicide plasmid, pCVD442. The respective suicide plasmids were first transformed into *E. coli* Sm10 λ *pir* and then transferred into *V. cholerae* via conjugation. Conjugants were purified on Sm- and Ap-containing agar plates to select for the integration of the plasmid into the *V. cholerae* chromosome, followed by growth on sucrose to select for the second recombination and obtain Ap^r colonies, in which an excision of the plasmid from the chromosome took place. The correct deletions were confirmed by PCR (data not shown). For construction of the expression plasmid pmshA, the oligonucleotides VC0409-SacI_fw and VC0409-XbaI_rv were used to amplify the respective gene (Table S3). The resulting PCR

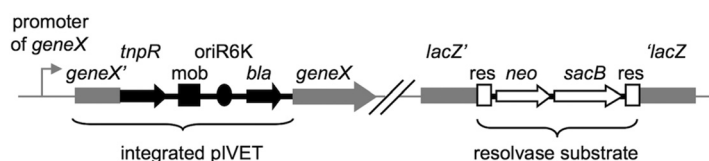


FIG 8 Illustration of the genetic components of RIVET. Chromosomal sequences are in gray, pIVET parts are in black, and the res cassette parts are in open shapes. The suicide vector pIVET is integrated into *V. cholerae* hypothetical *geneX* via homologous recombination, resulting in a merodiploid state in which *geneX* and *tnpR* (resolvase) are transcriptionally fused and controlled by the chromosomal promoter of *geneX*. The mobilization (*mob*), origin of replication (*oriR6K*), and Ap^r (*bla*) regions of pIVET, as well as the genes for Km^r (*neo*) and Suc^c (*sacB*) and the target sites of resolvase (*res*) of the res cassette, are indicated.

fragment was digested with the appropriate restriction enzymes and ligated into an identically digested IPTG-inducible plasmid, pMMB67EH. Ligation products were transformed into DH5 α *lpir*, and Ap^r colonies were characterized by PCR for the correct construct (data not shown).

Screening for *aec* genes. A slightly adapted version of the recombination-based *in vivo* expression technology (RIVET, Fig. 8) in combination with a library of 12,000 random transcriptional gene-*tnpR* fusions (16, 41) was used to identify genes induced during colonization of *C. elegans*, which was consequently renamed recombination-based in *C. elegans* expression technology (RICET). The original gene-*tnpR* fusion library was generated to identify genes induced during colonization of the mouse model and consequently constructed using LB broth at 37°C. To minimize the identification of differentially expressed genes solely caused by the specific growth requirements of *C. elegans*, the entire gene-*tnpR* fusion library was passaged once on NGM plates at 22°C prior to the screening for *aec* genes. Briefly, each pool was spread on NGM plates and incubated overnight at 22°C before the bacteria were collected from the NGM plate and purified on LB-Ap/Sm/Kn plates to remove all resolved strains. Finally, the NGM-passaged library was refrozen in 12 purified pools containing ~5,000 colonies (Ap^r/Sm^r/Kn^r) each. For the screening of *aec* genes, an aliquot of each pool of the library was spread in triplicate on LB-Ap/Sm/Kn plates, resuspended in LB broth, and adjusted to an optical density at 600 nm (OD₆₀₀) of 0.1. One hundred microliters of the suspension was spread in the middle of an NGM agar plate in a rectangular shape using a Drigalski spatula. After incubation overnight at 22°C, ~60 synchronized young adult worms were transferred onto the plate and allowed to graze for 16 h at 22°C. To remove bacteria attached on the outside of the nematode, the worms were then transferred onto an empty NGM agar plate for 1 h followed by a 1-min dip in 30% ethanol. Finally, batches of ~20 worms were collected in 1 ml saline, briefly vortexed, and homogenized using glass beads (1 mm in diameter; Roth) in combination with a PowerLyzer 24 (Mo Bio Laboratories, Inc.), applying 2,000 rpm for 1 min. Efficient removal of bacteria attached on the outside of the nematode and lysis of *C. elegans* worms were monitored by plating appropriate dilutions directly before and after homogenization on LB-Sm agar plates. Throughout the screen, less than 10 CFU/worm was observed before homogenization, while after homogenization ~10³ CFU/worm could be detected (data not shown). Serial dilutions were plated on Sm/Suc agar plates (1% tryptone, 0.5% yeast extract, 1.6% agar) to select for resolved strains. After a 2-day incubation at room temperature (RT), colonies were picked, patched in parallel on LB-Sm/Ap and LB-Sm/Km agar plates, and incubated overnight at 37°C. Ap^r and Km^s clones were screened for diverse gene-*tnpR* fusions via different PCR product sizes using the oligonucleotides IVET-1 and IVET-2 as described previously (37). The plasmids containing the gene-*tnpR* fusion (pIVETs) of 5 to 8 different resolved strains were recovered (Qiagen Spin Miniprep kit), and aliquots were sent to Agowa Genomics for sequencing with the oligonucleotide IVET-3 as previously described (17, 42). Sequences were compared to the *V. cholerae* N16961 genome database at the J. Craig Venter Institute with BLASTn (<http://blast.jcvi.org/cmr-blast/>). Gene annotations and operon predictions shown in Table S1 were determined by the KEGG database (<http://www.genome.jp/kegg/>) and the operon prediction database (<http://biocomputo2.ibt.unam.mx/OperonPredictor/>), respectively (43–46). Transcriptional fusions to any annotated open reading frame (ORF) within which *tnpR* had inserted in the same orientation were considered as described previously (16).

Reconstruction of fusion strains and determination of the resolution frequency. The reconstruction of unresolved *V. cholerae* fusion strains was carried out as described previously (38). For all resolution frequency quantifications, strains were grown overnight on LB-Sm/Ap/Km agar plates, resuspended in LB broth, and adjusted to an OD₆₀₀ of 5. Fifty microliters of this inoculum was spread in the middle of an NGM plate in a rectangular shape using a Drigalski spatula and air dried for a few minutes. To determine the resolution frequency *in vitro* (control condition), plates were incubated for 16 h at 22°C before bacteria were harvested and resuspended in LB broth. To determine the resolution frequency in *C. elegans*, ~60 synchronized young adult worms were transferred onto the plate and allowed to graze for 16 h at 22°C before the worms were purified from bacteria attached to the outside and homogenized as described above (see “Screening for *aec* genes”). The resolution frequencies *in vitro* as well as in *C. elegans* were determined by plating serial dilutions on LB-Sm/Ap and LB-Sm/Km agar plates. The resolution frequency is expressed in percent and calculated as follows: (Sm/Ap CFU – Sm/Km CFU)/(Sm/Ap CFU × 100), as described previously (16).

C. elegans survival assay. Survival assays were essentially performed as previously described (12, 13). Respective *V. cholerae* strains or *E. coli* OP50 was incubated at 37°C overnight in LB broth and

adjusted to an OD₆₀₀ of 1, and 100 μ l was spread on an NGM agar plate in a rectangular shape using a Drigalski spatula. After incubation overnight at 24°C, ~30 synchronized L4-stage *C. elegans* SS104 worms were transferred onto the plates. Plates were subsequently incubated at 24°C and scored for live worms every day. A worm was considered dead when it no longer responded to touch. Any worms that died as a result of migrating outside the bacterial rectangular lawn and getting stuck to the wall of the plate were excluded from the analysis.

C. elegans developmental assay. Respective *V. cholerae* strains or *E. coli* OP50 was incubated at 37°C overnight in LB broth and adjusted to an OD₆₀₀ of 1, and 100 μ l was spread on an NGM agar plate in a rectangular shape using a Drigalski spatula. In the case of strains harboring pMMB67EH or derivatives, NGM plates supplemented with IPTG (0.5 mM) and Ap (100 μ g/ml) were used. After incubation overnight at 24°C, ~20 synchronized young adult *C. elegans* SS104 worms were transferred onto the plate and allowed to lay eggs for 2 h before they were removed. This was defined as day 0, was allowed to control synchronized hatching, and resulted in similar numbers of eggs on different NGM agar plates (~30 eggs/plate). Plates were incubated at 24°C, and development into at least the L4 stage was monitored every 24 h for 10 days. The L4 stage was chosen as the criterion as it can be easily distinguished via microscopy from earlier stages by the presence of a vulva. Data are presented as percentage of worms in the L4 stage or later stages divided by the total number of live worms present on day 3. All worms moving or responding to touch were considered to be alive. During the developmental assay, the length of the worms was determined on day 4 using the ImageJ software from images of living worms, and colonization levels were quantified as described below (see “Quantification of bacterial colonization” for details).

Microscopy. For routine handling, developmental stage evaluation, and length determination of *C. elegans*, the Leica M60 or Motic SMZ-140-N2GG stereomicroscope was used. Nematodes on NGM plates were imaged using the Leica M60 stereomicroscope in combination with a Tucsen 3.0 MP microscope USB camera. Localization of *V. cholerae* in *C. elegans* was investigated by fluorescence microscopy using a Nikon Eclipse Ti-E inverted microscope equipped with a Nikon DS-Qi2 camera and Nikon Plan Fluor 40 \times objective (numerical aperture [NA], 1.30). All images were analyzed by Nis-Elements BR version 4.30.02 software. For localization studies, synchronized L4-stage *C. elegans* SS104 worms were passaged via NGM agar plates without bacteria to fast for approximately 4 h, before they were transferred onto NGM agar plates containing a rectangular lawn of GFP⁺ bacteria (prepared as described above) and incubated for 20 h at 24°C. Afterward, the nematodes were either directly prepared for microscopy or transferred to NGM agar plates seeded with *E. coli* OP50 without GFP expression for another 2 h to remove loosely attached bacteria inside the worms prior to microscopy. Alternatively, worms were allowed to lay eggs on NGM agar plates seeded with *V. cholerae* pgfp, and plates were subsequently incubated at 24°C for 4 days. Prior to microscopy, worms were transferred to NGM agar plates seeded with *E. coli* OP50 for 2 h. Subsequently, worms were immobilized using Na₂S₂O₈ (0.01%) and embedded in fluorescent mounting agent (Dako). Presence and localization of *V. cholerae* in *C. elegans* were investigated by fluorescence microscopy using a Nikon Eclipse Ti-E inverted microscope equipped with a Nikon DS-Qi2 camera and Nikon Plan Fluor 10 \times (NA, 0.3) or 40 \times (NA, 1.30) objective. GFP was detected using an excitation of 400 nm and emission of 500 to 550 nm. All images were analyzed by Nis-Elements BR version 4.30.02 software. A signal intensity inside the worm at least 10-fold above background (surrounding the worm) was defined as positive signal. The statistical significance of associations between the incidences of positive signals observed in *C. elegans* grazing on either *V. cholerae* WT or mutant was assessed by the odds ratio using the Fisher exact test (Prism5; GraphPad Software, USA).

Quantification of bacterial colonization. Colonization was determined for living *C. elegans* SS104 worms on day 4 of the developmental assay (see “*C. elegans* developmental assay”) or for synchronized L4-stage *C. elegans* SS104 worms grazing for 20 h on *V. cholerae* WT or Δ *mshA* mutant (see “Microscopy”). Hence, worms were allowed to lay eggs on NGM agar plates seeded with either *V. cholerae* WT or Δ *mshA* mutant, and plates were subsequently incubated at 24°C for 4 days. Alternatively, worms were synchronized on *E. coli* OP50 for 3 days and subsequently transferred onto NGM agar plates seeded with *V. cholerae* WT or Δ *mshA* mutant for 20 h.

In all cases, worms grazing on *V. cholerae* were transferred to NGM agar plates seeded with *E. coli* OP50 for another 2 h and subsequently onto an empty NGM plate for 2 min to remove loosely attached bacteria, followed by a 1-min dip in 30% ethanol to eliminate bacteria attached to the exterior surface of the worm. Then, 7 to 12 worms were collected in 1 ml LB broth, briefly vortexed, and homogenized using glass beads (1 mm in diameter; Roth) in combination with a PowerLyzer benchtop bead-based homogenizer (Mo Bio Laboratories, Inc.), applying 2,000 rpm for 1 min. Remaining bacteria attached on the outside of the nematode were monitored by plating appropriate dilutions directly before homogenization on LB/Sm agar plates, while colonization levels of the worms were determined by plating appropriate dilutions of the homogenized worms on LB/Sm agar plates. In all cases, the CFU before homogenization was at least 10-fold lower than the CFU after homogenization, suggesting efficient removal of bacteria attached to the exterior of the worm. Results are given by CFU/worm calculated as follows: (CFU after homogenization – CFU before homogenization)/number of worms.

Statistical analysis. Unless stated otherwise, the data are presented as medians with interquartile ranges. Data were analyzed using the Mann-Whitney U test for single comparisons or the Kruskal-Wallis test followed by *post hoc* Dunn’s multiple-comparison test, and differences were considered significant for *P* values of <0.05.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/mSphereDirect.00238-18>.

FIG S1, TIF file, 1 MB.

FIG S2, TIF file, 0.5 MB.

FIG S3, TIF file, 2.8 MB.

FIG S4, TIF file, 2.7 MB.

TABLE S1, DOCX file, 0.03 MB.

TABLE S2, DOCX file, 0.03 MB.

TABLE S3, DOCX file, 0.02 MB.

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